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Neurotoxic action of the Cry3A toxin on the Colorado potato beetle, *Leptinotarsa decemlineata*, with permethrin as a comparison

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Abstract: Spontaneous discharges of action potentials (AP) of neuromuscular preparations in the leg and fore- or mid-gut of the Colorado potato beetle, *Leptinotarsa decemlineata* were recorded in situ under the treatment of oral injection of Cry3A toxin and permethrin insecticide. Both of the two chemicals exhibited rather specific electrophysiological symptoms in the preparations: they evoked groups of action potentials with highly increased frequencies in the early stage, and led to long period of interspike resting, the duration of which increased as intoxication proceeded. Permethrin typically caused extremely high frequency discharges (overshooting) in the leg preparations and very short interspike resting periods, but the Cry3A toxin only exhibited these effects in the gut preparations. Also Cry3A toxin increased excretion dramatically and decreased feeding of the treated beetles. These results indicated that the nerve-muscle system in the gut appears to be much more sensitive to Cry3A toxin than that in the leg and the primary neurotoxic or myotoxic action of Cry3A, causing a disturbance of the gut activity, is an important mechanism of its insect poisoning consequence.

Key words: *Leptinotarsa decemlineata*; Cry3A toxin; permethrin; electrophysiology

1 INTRODUCTION

The bacterium *Bacillus thuringiensis* (Bt) produces intracellular parasporal portentous crystals that are highly toxic to a variety of insects. Cry3A toxin gene has transferred to several kinds of plants (Gill *et al.*, 1982; Denolf *et al.*, 1993). Commercialization and large-scale production of the Bt-transgenic plants is rapidly raising the importance of the endotoxins in agricultural production (Bergelson *et al.*, 1998). However, its increased use and commercial exploitation has been restricted by a number of problems including the risk of rapid pest adaptation and the lack of knowledge of its precise mode of action (Ferco, 1992; Whalon, 1993; Hoy and Head, 1995; Tabashinik, 1997).

In spite of significant efforts towards the study of the toxin's mode of action, the realization of toxicological mechanism is not entirely known. Investigations had revealed that the activated toxin appears to insert into the plasma membrane, and interact with a receptor or ion channel in the brush border membrane vesicle (BBMV) of the columnar cells of the midgut, forming a pore. These pores were supposed to increase the ion conductance of the epithelial cell apical membrane, leading to disruption of the electrical Na^+, K^+, and pH gradients. Osmotic swelling and cell lysis resulted. Some authors have suggested that midgut epithelial damage is the cause of paralysis and/or death of the Bt-sensitive insects (Singh *et al.*, 1986). Recent work on Bt mode of action has been concentrating on the formation and conductance of the pores. Confirmation of pore formation has been demonstrated in several model membranes (Denolf *et al.*, 1993). The influx of monovalent cations (Na^+, K^+) induced by the toxin have been observed in insect-cultured cell systems. A similar effect on ion transportation with the pyrethroid insecticides was proposed (Singh and Gill, 1985; Cooper *et al.*, 1998). Other reports have showed that the muscular system appears to be more sensitive to the toxin than the central nervous system.

To demonstrate the neurotoxic action of the Cry3A toxin, we conducted a comparative study on the toxin in comparison with a typical neurotoxicant permethrin. Electrophysiological effects of both toxins on muscle systems in the hind-leg and fore-or mid-gut of the Colorado potato beetle, *Leptinotarsa decemlineata* (Say) were recorded and compared.

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2 MATERIALS AND METHODS

2.1 The insects

Insects used in the electrophysiological study are adults of *Leptinotarsa decemlineata*（Say）collected from Wooster， Ohio， maintained in Department of Entomology， The Ohio State University， which are reared in greenhouse on potted potato plants covered with a plastic cage. Female adults in standard body size were selected by visual inspection and held for 1 h without food in a petri dish on moist filter paper before treatment. The early 3rd instar larvae were selected for the bioassay. Larvae used in every replication are collected from the same family and held for 1 h without food in a petri dish on moist filter paper before treatment.

2.2 The toxins

Cry3A toxin was provided by the Monsanto Company kept in the following solution： 25 mmol/L Na_2CO_3， 0.1 mmol/L $NaHCO_3$， pH 10.5. High（0.2 μL × 25 mmol/L/larva）and low dosage（0.2 μL × 6.25 mmol/L/larva）of the toxin were set for the treatment. Permethrin（Ambush， emulsifiable concentrate， ICI， Philadelphia， PA）was selected as a representative pyrethroid， and was diluted in D. I water for the treatment. The Cry3A solution was kept in the refrigerator， at approximately 5℃， other solutions were prepared before use. The Cry3A toxin was diluted with the buffer containing 25 mmol/L Na_2CO_3， 0.1 mmol/L $NaHCO_3$，（pH 10.5）. The toxin solutions（0.2 μL）were injected into the foregut through the mouth of the insect using a electronic microinjector.

2.3 Insect assays

For preliminary assays， larvae were provided standard potato foliage and observed for symptoms for 120 hours after injecting the 2 μL of test solution. Bioassays on 45 3rd instars for doses ranging from 0.0125 to 0.2 μg/L and a buffer control resulted the following equation： $Y = 3.5249 + 0.2063\ X$（r = 0.9281）So the LC_{50} value is 38.8505 ± 11.9604.

To record excrements of the beetles， 30 – 40 4th instar treated larvae were placed on filter papers in plastic Petri dishes. The Petri dishes were kept in a chamber with high humidity. The filter papers are replaced at regular time interval and treated with 0.001% ninhydrin in acetone solution. After being oven-dried for 5 min at 100℃， the average area of the stained spots were measured and calculated by using a thin layer chromatogram scanner.

To examine the multi-action potentials of the foregut， all the legs and the dorsal cuticle of abdomen were removed. The beetles were mounted with insect pins in a groove in a polyester board. The recording electrode（platinum， bare， ϕ0.1 mm）was carefully inserted into the mouth under dissection microscope. To record action potentials in the mid-gut， the gut was carefully cut behind the crop， and the end of the midgut was carefully pulled out and placed by the side of the cuticle. The recording electrode was inserted into the cavity of the gut. Both the reference electrodes were set in the hemolyph beneath the hind-gut. To keep the gut moist， drops of physiological saline（175 mg $CaCl_2$； 500 mg KCl； 75 mg KH_2PO_4； 250 mg $MgSO_4$； 10 mg NaCl； 438 mg $NaHCO_3$； 75 mg Na_2HPO_4）were applied topically to the gut at intervals of 30 min.

To record action potentials in the leg， the beetle was dorsal side down in a groove on a polyester board， and one hind leg was fixed tightly with insect pins and stick glue. A fine platinum recording electrode（head sharpened， ϕ0.01 mm）was carefully inserted into the femur cuticle. The reference electrode was inserted into the coxal membrane. To prevent leaking of hemolyph fluid， the cuticle was sealed around the electrode with pure Vaseline. All the action potentials were amplified through a bio-amplifier and monitored with a Tektronix oscilloscope， connected in parallel to a computer using a data acquisition and analysis DI-90 I/D card. The average frequency was calculated by counting the number of spikes in 100 ms. All devices used in these experiments were shielded from electric interference with aluminum window screen. All experiments were carried out at a temperature of（25 ± 2）℃.

3 RESULTS

3.1 Symptoms of the intoxicated beetles

The behaviors indicating poisoning follow from the mode of action or resistance. Our observations on the intoxicated Colorado potato beetle larvae and adults were concentrated on changes in movement and excretion following injection of Cry3A toxin solution. The highly intoxicated insects， treated with the high dose， were characterized by decrease in movements and increase in excretion throughout the observation period.

Larvae regurgitated and did not feed. Less intoxicated insects， treated with the low dose， decreased movement during the first hour after toxin injection， and increased movement， secretion of saliva， regurgitation 1 – 6 h after injection. No significant differences in locomotion rates were observed in the first hour after treatments were injected. Beetles in all three treatments were moving at an average rate of 0.6 – 1.0 mm/s. During the second hour after treatment， however， locomotion rate of both the buffer control and the low-dose Bt treated beetles increased whereas that of the high dose treated beetles decreased. This dose-dependent response to Bt ingestion is similar to that observed in previous research using frequency of movement rather than movement rate as the measure of activity (Hoy *et al*. unpublished). From 6 – 24 h after injection the movement of larvae treated with either dose decreased relative to controls. The larvae appeared extremely sluggish, ceased feeding, and died from starvation and desiccation finally. In contrast, the typical symptoms of permethrin-intoxicated beetles were greatly increased movement, but no significant change in excretion (Fig. 1).

The average total excretion by Cry3A treated larvae, which consisted of both regurgitation and lapaxis, during the first 24 h was more than three times that of the buffer treated control. After 24 h, excretion, mostly regurgitation, kept at a much higher level than the control. Disorder in the gut nerve-muscle system may partly contribute to starvation and desiccation in treated larvae.

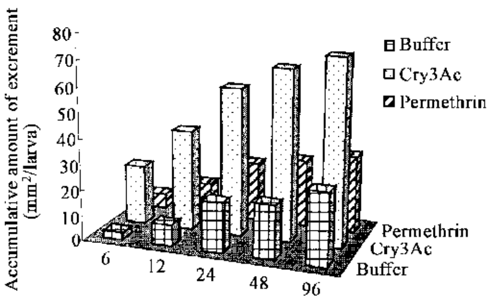

Fig. 1 Average amount of the excrements of high (0.2 μL × 1 mg/mL/head) and low dose (0.2 μL × 0.01 mg/mL/head) Cry 3A toxin and permethrin treated 4th instar larvae of *L. decemlineata*

3.2 Effects upon the midgut muscle action potentials

In the midgut preparations of the beetles injected with buffer solution, a single spike or group of 3 – 6 spikes occurred in regular intervals. The average frequency amounted to approximately 20 – 48 spikes/min, amplitude 1 – 9 mV, and interspike interval 2 – 5 s (Fig. 2B). In the midgut preparations injected with high dose, a much toxin higher frequency of repetitive discharges were recorded approximately 5 min after the injection of the Cry3A endotoxin The average firing frequency was approximately 600 – 1 000 spikes/min, amplitude approximately 1 – 6 mV (Fig. 2B). After 2 – 5 min high frequency discharge, the frequency decreased and the duration of interspike intervals gradually increased. Fifteen min after the injection, only low frequency (approximately 100 – 400 spikes/min) and low amplitude (0.2 – 2 mV) groups of spikes were recorded, interspike intervals lasted as long as 15 min. In preparations injected with low dose toxin, high frequency (200 – 400 spikes/min) and high amplitude (1 – 26 mV) action potential firings appeared for a short period and gradually decreased from 5 min until 2 h after the injection.

Discharge frequency was characterized by a decrease in firing in the high dose treatment and an increase in the low dose treatment relative to the buffer control during the interval from 30 – 40 min after treatment (Fig. 2D). The duration of firing was longer than that seen in the high dose treatment, and the interspike interval was shorter. In contrast, little influence on action potentials in the midgut was observed in the permethrin treatment. The firing frequency and amplitude slowly increased 5 min after the injection. Occasional groups of spike firing were found throughout the observation. The frequency of action potentials was much lower, however, than that of Cry3A toxin treatments (Fig. 2C).

3.3 Effects upon the foregut action potentials

The action potential recorded from the foregut muscle was clearly different from those recorded in the midgut. The action potentials recorded in the beetles injected with buffer were at a frequency at 250 – 350 spikes/min, which was concededly higher. Recordings tended to be composed of rhythmic, single potentials of uniform amplitude (3 – 6 mV). Groups of spikes or

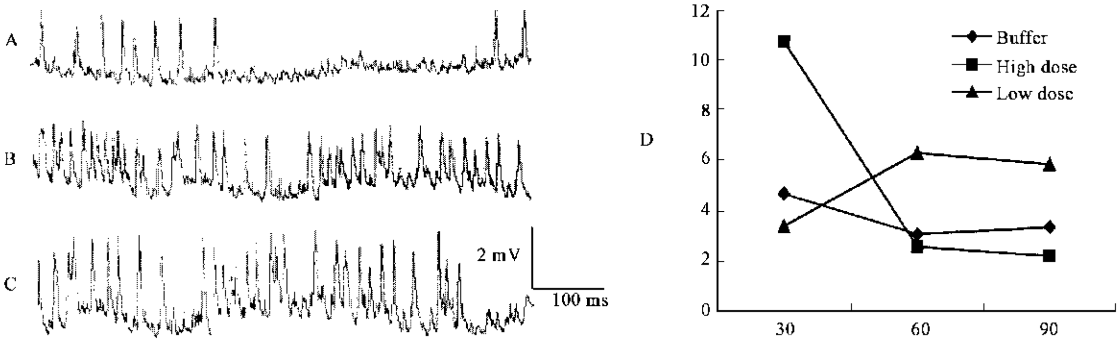

Fig. 2 The samples of Cry3A toxin-and permethrin-induced spontaneous action potentials recorded from the midgut preparations of *Leptinotarsa decemlineata* adults

A. discharges occurred in the control beetle (injected with buffer solution); B. repetitive discharges evoked by Cry3A; C. repetitive discharges caused by permethrin treatment; D. Frequency (spikes/100ms).

spikes with higher amplitude were rarely recorded（Fig. 3A）. Cry3A endotoxin injection resulted in repetitive action potentials，with groups of spikes lasting 2 – 3 s，a discharge frequency of approximately 1 000 – 1 500 spikes/min and amplitude of 0.5 – 6 mV（Fig. 3B）. The interval between discharges varied from 1 – 5 s. Typical an extremely high frequency discharges（overshooting）began 5 – 10 min after injection，lasting 1 – 3 min. The high frequency discharge frequently reoccurred in high frequency groups of discharges of progressively decreasing amplitude. These were quite similar to those groups of high frequency discharge caused by permethrin. Intervals between discharges in the permethrin treatment were much shorter（0.1 – 2 s），however，and amplitudes were much higher（4 – 16 mV）than those in Cry3A（Fig. 3C）.

Some much higher firing frequency samples can be found in the gut system during the early 30 min. And some highest frequency bursts of spikes（overshooting）occurred in the high dose treatment. It indicates the occurrence of the toxic effects on the gut system take place in advance. There seemed one kind of asynchronous relation between the behavioral response and the midgut system response. High frequency led to increasing of the movement，while the extreme high frequency caused long term inhibition of discharge and decreasing of the movement. These may infer that the primary disorder in the gut nerve-muscle system.

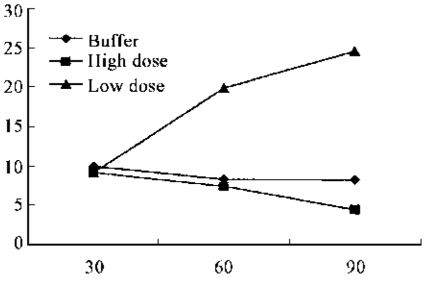

Fig. 3　The samples of Cry3A toxin-and permethrin-induced spontaneous action potentials recorded from the foregut preparations of *Leptinotarsa decemlineata* adults

A. control beetle（injected with buffer solution）; B. discharges evoked by the Cry3A toxin; C. discharges caused by permethrin treatment; D. Frequency（spikes/100 ms）.

3.4　Effects upon the leg muscle action potentials

Action potential change after Cry3A injection took a longer time in the leg muscle preparations than in the gut. Change in spike patterns occurred 15 – 30 min following the injection. The frequency of discharges increased gradually to a very regular high frequency bursting（500 – 1 000 spikes/min），lasting 1 – 3 min each. Such high frequency bursts were not recorded from buffer injected beetles. Amplitude of the discharges（4 – 16 mV）however，remained constant（Fig. 4B）. In contrast，continuous high frequency firing occurred after the permethrin injection，lasting up to the entire recording period with short periods of resting. Extreme high frequency overshooting was recorded during the early 30 min – 1 h（Fig. 4C）. The excitement effect of permethrin on the leg neuro-muscle system was much stronger than that of Cry3A，and is known to increase locomotion of the treated insects.

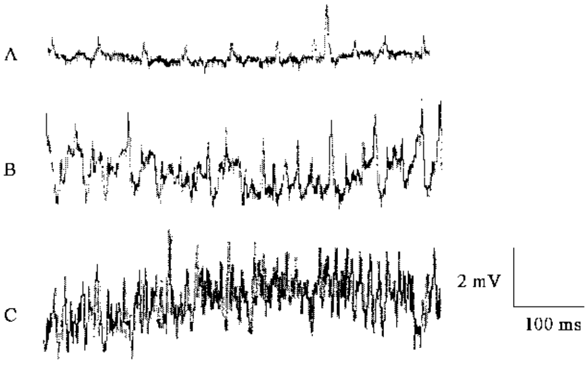

Fig. 4　The samples of Cry3A toxin-and permethrin-induced spontaneous action potentials recorded from the leg preparations of *Leptinotarsa decemlineata* adults

A. control beetle（injected with buffer solution）; B. discharges evoked by the Cry3A toxin; C. discharges caused by permethrin treatment.

4　DISCUSSION

Although pyrethroid insecticides mainly influence the insect central nervous system，the behavioral effects on treated insects are more closely correlated with the peripheral nervous system，which may contain primary target sites（Adams and Miller 1998）. The foregut and anterior portion of the midgut are innervated from the frontal and ingluvial ganglia，the intervening recurrent and oesophageal nerves，and the ingluvial nerve. The stomatogastric nervous system controls the muscles of the gut. Motor nerves here control the release of secretion. In this study we found that the Cry3A endotoxin resulted in similar effects to pyrethroids on the neuromuscular systems in both the leg and the gut. Cry3A also resulted in midgut effects not associated with pyrethroids，the effects led to changes in movement and excretion. Therefore Cry3A may produce a neurotoxic effect similar to permethrin on the motor nerves of the treated insect.

Early experiments demonstrated that Cry endotoxin damages the muscle faster than it disrupts nerve function. CryIA caused a rapid and irreversible depolarization of the membrane of midguts of gypsy moth（*Lymantria dispar*）and silkworm（*Bombyx mori*）larvae（Peyronnet *et al*.，1997）. Cry endotoxin also caused increased of the K^+ permeability has been verified in a model membrane system（Singh and Gill，1985，Cooper *et al*.，1998）. Primary myotoxic action of the Cry endotoxin was proposed. In arthropod muscle membranes，Ca^{++} channels and K^+ channels are responsible for generating action potentials which control gut movements. It is，therefore，possible that Cry3A toxin interfered with at

least one of the two channels.

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Cry3A 毒素和氯菊酯对马铃薯甲虫神经-肌肉系统的电生理作用比较

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摘要：对 Cry3A 毒素和氯菊酯杀虫剂经口注射处理的马铃薯甲虫的前肠、中肠和腿节样本的神经肌肉自发动作电位发射进行了记录。两种化合物均可呈现很典型的神经电生理症状：在初期阶段，它们均引起连放动作电位的发放程度大幅增加，而发放间隔期延长，且随着中毒加深而拉长。氯菊酯可在腿节样本引起典型高频爆排，表现出间隔非常短的静息期特征，但 Cry3A 毒素只在肠道样本中表现上述特征。而且 Cry3A 毒素可使处理甲虫的呕吐物大幅增加，而取食减少。这些结果显示处理虫的肠道神经肌肉系统较腿部神经肌肉系统对 Cry3A 毒素更敏感，Cry3A 毒素最初的神经毒性或细胞毒性作用引起肠道活动的紊乱是其昆虫毒性作用的重要机制。

关键词：马铃薯甲虫；Cry3A 毒素；氯菊酯；电生理

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